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Coronavirus TGEV Evades the Type I Interferon Response through IRE1α-Mediated Manipulation of the miR-30a-5p/SOCS1/3 Axis

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Running title: TGEV escapes IFN-I via the miR-30a-5p/SOCS pathway

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Abstract

In host innate immunity, type I interferons (IFN-I) are major antiviral molecules, and coronaviruses have evolved diverse strategies to counter the IFN-I response during infection. Transmissible gastroenteritis virus (TGEV), a member of the alphacoronavirus family, induces endoplasmic reticulum (ER) stress and significant IFN-I production after infection. However, how TGEV evades the IFN-I antiviral response despite the marked induction of endogenous IFN-I has remained unclear. IRE1α, a highly conserved ER stress sensor with both kinase and RNase activities, is involved in the IFN response. In this study, IRE1α facilitated TGEV replication via downmodulating the host miR-30a-5p abundance. miR-30a-5p normally enhances IFN-I antiviral activity by directly targeting the negative regulators of JAK-STAT, SOCS1 and SOCS3. Furthermore, TGEV infection increased SOCS1 and SOCS3 expression, which dampened IFN-I antiviral response and facilitated TGEV replication. Importantly, compared with mock infection, TGEV infection *in vivo* resulted in decreased miR-30a-5p levels and significantly elevated SOCS1 and SOCS3 expression in piglet ileum. Taken together, our data reveal a new strategy used by TGEV to escape the IFN-I response by engaging the IRE1α-miR-30a-5p-SOCS1/3 axis, thus improving our understanding of how TGEV escapes host innate immune defenses.

Key words: Transmissible gastroenteritis virus (TGEV), IRE1α, miR-30a-5p, SOCS, Type I interferon

Importance: Type I interferons (IFN-I) play essential roles in restricting viral infections. Coronavirus infection induces ER stress and the interferon response, which reflects different adaptive cellular processes. An understanding of how coronavirus-elicited ER stress is actively involved in viral replication and manipulates the host IFN-I response has remained elusive. Here, TGEV inhibited host miR-30a-5p via the ER stress sensor IRE1α, which led to the increased expression of negative regulators of JAK-STAT signaling cascades, namely, SOCS1 and SOCS3. Increased SOCS1 or SOCS3 expression impaired the IFN-I antiviral response, promoting TGEV replication. These findings enhance our understanding of the strategies used by coronaviruses to antagonize IFN-I innate immunity via IRE1α-mediated manipulation of the miR-30a-5p-SOCS axis, highlighting the crucial role of IRE1α in innate antiviral resistance and the potential of IRE1α as a novel target against coronavirus infection.

Introduction

Endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) are common consequences of coronavirus infection (1-5). Our groups and others have demonstrated that coronaviruses, including severe acute respiratory syndrome coronavirus (SARS-CoV), infectious bronchitis virus (IBV), porcine epidemic diarrhea virus (PEDV), and transmissible gastroenteritis virus (TGEV), are all capable of inducing significant ER stress following infection and simultaneously trigger multiple UPR pathways to restore ER homeostasis (1, 5-8). During ER stress, inositol-requiring enzyme 1 α (IRE1α) is activated by oligomerization and autophosphorylation (9-11). IRE1α activation initiates diverse downstream signaling of the UPR either through splicing of X-box binding protein 1 (XBP1) or through posttranscriptional modifications via IRE1α-dependent mRNA degradation (RIDD) (9, 12). IRE1α regulates genes involved in protein entry into the ER, folding, glycosylation, and ER-associated degradation (ERAD) to facilitate ER homeostasis (11, 13). Moreover, IRE1α degrades ER-localized mRNAs via RIDD to reduce the burden of protein entry into the ER (14). In addition to degrading mRNA, activated IRE1α has recently been demonstrated to cleave precursor microRNAs (pre-miRNAs) and to degrade miRNAs during noninfection-derived ER stress (15, 16). Given the crucial roles of IRE1α signaling in cellular fate determination, many RNA viruses, such as influenza A virus (IAV) and Japanese encephalitis virus (JEV), employ IRE1α signaling to facilitate their replication (17, 18). However, whether and how IRE1α specifically modulates coronavirus replication are not well established.

Type I interferons (IFN-I) (IFN-α/β) play crucial roles in host antiviral responses. Upon viral infection, host cells react quickly to the invading viruses by synthesizing and secreting IFN-I. Binding of IFN-I to its receptor (interferon alpha/beta receptor, IFNAR) results in activation of Janus family kinases (JAKs) and the subsequent activation of signal transducer and activator of transcription (STAT) signaling cascades, thereby inducing the expression of

IFN-stimulated genes (ISGs) (19). However, over the course of the long evolutionary competition between viruses and host cells, coronaviruses have evolved diverse mechanisms to counteract the IFN-I response (20-23). At least 11 viral proteins of coronavirus SARS-CoV and PEDV have been identified as IFN-I antagonists (20, 23, 24). By contrast, TGEV induces high levels of IFN-I *in vivo* and *in vitro* after infection (25-27). Despite a wealth of knowledge regarding how TGEV triggers IFN-I production, how TGEV counters the antiviral activity of IFN-I has not been fully elucidated.

microRNAs (miRNAs) are a large family of short (19-24 nucleotides) noncoding RNAs that regulate gene expression posttranscriptionally through translational repression and/or mRNA degradation by binding their seed regions to complementary sites present in the 3' untranslated region (UTR) of target genes (28, 29). Given the critical roles of miRNAs in regulating gene expression, unsurprisingly, viruses take advantage of host miRNAs to target vital components of the IFN-I response and impair IFN-I antiviral activity for optimal infection (28, 30, 31). JEV evades IFN-I and enhances viral infection by downregulating the expression of miR-432, which directly targets the suppressor of cytokine signaling protein 5 (SOCS5), a negative regulator of the JAK-STAT1 signaling cascade (32). Porcine reproductive and respiratory syndrome virus (PRRSV) dampens the JAK-STAT signaling of IFN-I to facilitate its replication via upregulating host miR-30c, which directly targets JAK1 (30). However, the potential role of miRNAs in the coronavirus escape from the IFN-I response has remained elusive.

Aberrant miRNA expression is integrally related to the progression and pathogenesis of diseases (30, 33, 34). Although we have gained considerable insights into aberrant miRNA expression by cis-regulatory elements and trans-acting factors caused by viral infection, the contribution of the virus-induced UPR to aberrant miRNA expression has rarely been investigated. Recent studies have shown that activated IRE1α affects cell fate by directly degrading a subset of host miRNAs (miR-17, miR-34a, miR-96, and miR-125b)

under persistent ER stress induced by chemicals or noninfectious diseases (15, 35). However, whether and how viruses exploit IRE1α to manipulate miRNA expression for optimal viral infection remain unknown.

In this study, TGEV infection downregulated the expression of host miR-30a-5p via virally triggered IRE1α-mediated UPR induction. miR-30a-5p suppressed TGEV infection by enhancing the IFN-induced antiviral signaling pathway by directly targeting the negative regulators of IFN signaling, SOCS1 and SOCS3. Moreover, TGEV infection *in vivo* suppressed miR-30a-5p expression and significantly elevated the expression of SOCS1 and SOCS3 in the ileum. Altogether, these data contribute new insights into the roles of IRE1α in regulating the innate immune response and help to explain how TGEV escapes host IFN-I innate immunity.

Results

TGEV infection downregulates miR-30a-5p expression

The host miR-30 family (five members: miR30a-e) plays important roles in cancers and viral infections (30, 34, 36, 37). We recently reported that miR-30a-5p, a member of the miR-30 family, is downregulated and inversely correlated with the levels of ER stress in renal cancer, indicating that ER stress might inhibit miR-30a-5p expression (34). To assess whether ER stress suppresses the expression of miR-30a-5p, we initially analyzed the levels of miR-30a-5p in swine testicular (ST) cells following treatment with the ER stress inducer thapsigargin (Tg). Tg treatment substantially diminished the abundance of miR-30a-5p and exhibited a dose-dependent suppression (Fig. 1A), indicating that Tg-derived ER stress reduces miR-30a-5p abundance. Our lab and others have shown that similar to other coronaviral infections, TGEV infection triggers significant ER stress and initiates all three UPR pathways (1, 8). To explore whether miR-30a-5p could be regulated by TGEV infection, we initially monitored miR-30a-5p expression in ST cells after TGEV infection with different multiplicities of infection (MOIs). Compared with mock infection,

151 TGEV infection significantly reduced the levels of miR-30a-5p at 24 h
152 postinfection (hpi) and displayed a MOI-dependent response (Fig. 1B). To
153 determine the stage at which miR-30a-5p suppression by TGEV infection
154 occurs, we analyzed miR-30a-5p expression at different time points after
155 TGEV infection. TGEV infection with a MOI of 1 caused a typical cytopathic
156 effect (CPE) including syncytium in ST cells at 24 hpi and resulted in
157 approximately 35% cell death at 48 hpi. miR-30a-5p reduction occurred after
158 12 hpi and then gradually decreased up to 48 hpi (Fig. 1D), indicating that
159 TGEV infection decreases miR-30a-5p abundance at the late stage of infection.
160 TGEV infection in ST cells was confirmed by quantifying the viral genomes
161 (Fig. 1C and 1E). TGEV primarily infects villous epithelial cells in the small
162 intestine *in vivo* and causes watery diarrhea. To assess whether TGEV
163 infection also decreases miR-30a-5p expression *in vivo*, we quantified
164 miR-30a-5p expression in piglet ilea at 48 hpi. TGEV infection resulted in an
165 approximately 5-fold reduction in miR-30a-5p abundance in the ileum *in vivo*
166 (0.021±0.003) compared with that in uninfected control ileum (0.096±0.016)
167 (p<0.01) (Fig. 1F). TGEV infection in the ileum was confirmed by quantifying
168 TGEV RNA (Fig. 1G). These results demonstrate that TGEV infection
169 decreases miR-30a-5p expression.
170
171 **IRE1α-mediated UPR induction inhibits miR-30a-5p expression**
172 We next investigated the mechanisms responsible for the suppression of
173 miR-30a-5p by TGEV infection. In recent studies, IRE1α, a highly conserved
174 ER stress sensor comprising a protein kinase and RNase, has the ability to
175 degrade miRNAs in addition to degrading mRNA under ER stress (35, 38, 39).
176 IRE1α activation by TGEV infection was assessed by real-time polymerase
177 chain reaction (RT-PCR) and PstI digestion, which showed a recognition site
178 located within the 26-nt region of XBP1 cDNA removed by IRE1α-mediated
179 splicing, as previously described (12, 40). Consistent with previous results for
180 the coronavirus mouse hepatitis virus (MHV) (41), TGEV infection caused

substantial cytoplasmic cleavage of the XBP1u transcript into the XBP1s transcription factor starting at 24 hpi, indicating that IRE1α is activated by TGEV infection (Fig. 2A). IRE1α activation was further confirmed by analyzing the expression of the XBP1s downstream target gene, namely, ER-localized DnaJ homologue 4 (ERdj4) (Fig. 2B). The activity of IRE1α (the ratio of spliced XBP1 DNA to total XBP1u DNA) was inversely correlated with the levels of miR-30a-5p expression (Fig. 2A and 1D) (R=0.933, p<0.01). The decrease in miR-30a-5p expression primarily occurred within 24-48 hpi, the period in which significant IRE1α activation was triggered by TGEV infection (Fig. 1D and 2A). These findings suggest that TGEV-induced IRE1α activation may involve decreased miR-30a-5p expression during TGEV infection. To demonstrate that activated IRE1α is responsible for the downregulation of miR-30a-5p, we monitored the expression of miR-30a-5p in TGEV-infected or Tg-treated cells after inhibiting IRE1α function with 4μ8c, a highly specific and selective inhibitor of the RNase activity of IRE1α (14). The effective blockage of IRE1α RNase function by 4μ8c was confirmed by PCR analysis of XBP1 cleavage (Fig. 2C). The inhibition of IRE1α RNase by 4μ8c almost completely abolished the suppression of miR-30a-5p by TGEV or Tg (Fig. 2D and 2E). To further verify the contribution of IRE1α to miR-30a-5p expression, we knocked down IRE1α expression by specific small interfering RNAs (siRNAs), and the efficiency of IRE1α knockdown was confirmed by Western blotting (Fig. 2F). The silencing of IRE1α by siRNAs significantly rescued the decreased miR-30a-5p induced by TGEV infection (Fig. 2G) or Tg treatment (Fig. 2H) (p<0.05). In addition, the efficiency of miR-30a-5p restored by IRE1α siRNAs was correlated with the knockdown efficiency of IRE1α siRNAs (Fig. 2F-2H). Taken together, these results show that activated IRE1α reduces miR-30a-5p expression.

miR-30a-5p suppresses TGEV propagation

In agreement with our previous results, compared with control dimethyl

211 sulfoxide (DMSO) treatment, blocking IRE1α with 4μ8c significantly reduced
212 TGEV replication, as revealed by measuring the quantities of viral genomes
213 (Fig. 3A) and infectious virions (Fig. 3B) (8). Consistent with 4μ8c treatment
214 results, knockdown of IRE1α by siIRE1α#3, which was the most efficient at
215 silencing IRE1α, significantly reduced the TGEV genome quantity and titer.
216 Both siIRE1α#1 and siIRE1α#2 also exhibited a tendency to decrease TGEV
217 propagation in ST cells but not significantly because of silencing efficiency (Fig.
218 3C and 3D). The observed levels of TGEV reduction were correlated with the
219 knockdown efficiency of IRE1α siRNAs (Fig. 2F, 3C and 3D). These data
220 demonstrate that the IRE1α pathway promotes TGEV replication.

221 Since TGEV replication was reduced after blocking IRE1α signaling
222 cascades by 4μ8c or IRE1α-specific siIRE1α#3, which rescued the
223 IRE1α-mediated downregulation of miR-30a-5p expression, we reasoned that
224 miR-30a-5p may inhibit TGEV infection. To investigate the role of miR-30a-5p
225 in TGEV propagation, we monitored TGEV infection in ST cells after
226 transfecting miR-30a-5p mimics 24 h prior to infection. Compared with NC
227 mimics, miR-30a-5p mimics decreased the TGEV genome quantity and
228 progeny viral titer by up to 17-fold. The inhibition of TGEV by preexisting
229 miR-30a-5p mimics was dose dependent, and TGEV infection (MOI=0.01) was
230 substantially reduced by 80 nM and 160 nM miR-30a-5p mimics (p<0.001) (Fig.
231 3E and 3F). miR-30a-5p mimics also significantly decreased TGEV infection
232 when a MOI of 1 was used (data not shown). The suppression of TGEV
233 infection by miR-30a-5p was further confirmed by a TGEV nucleocapsid
234 protein immunofluorescence assay (IFA) (Fig. 3G). To determine the phase at
235 which preexisting miR-30a-5p overexpression suppresses TGEV infection, we
236 measured TGEV infection at different time points in the presence of
237 miR-30a-5p overexpression. miR-30a-5p significantly suppressed TGEV
238 replication at 24 and 36 hpi, indicating that miR-30a-5p-mediated reduction in
239 TGEV replication occurs at the late stage of TGEV infection (Fig. 3H).
240 Furthermore, the specific suppression of endogenous miR-30a-5p in ST cells

by the miR-30a-5p inhibitor boosted TGEV infection compared with that by the inhibitor NC (Fig. 3I). These results demonstrate that miR-30a-5p suppresses TGEV infection. To verify whether IRE1α facilitates TGEV replication by manipulating miR-30a-5p expression, we analyzed TGEV production in ST cells transfected with a miR-30a-5p inhibitor in the presence of 4μ8c at 1.0 MOI of TGEV. The suppression of endogenous miR-30a-5p by the miR-30a-5p inhibitor significantly abrogated 4μ8c-mediated TGEV suppression compared with that by the DMSO mock control (Fig. 3I), indicating that IRE1α facilitates TGEV infection by manipulating miR-30a-5p expression. Taken together, our data indicate that IRE1α enhances TGEV replication by decreasing miR-30a-5p abundance.

miR-30a-5p enhances IFN-I antiviral signaling cascades rather than IFN-I induction

Next, we explored the mechanisms responsible for miR-30a-5p-mediated TGEV inhibition. TGEV efficiently replicates in cells despite significant amounts of endogenous IFN-I production after infection (25, 26), indicating that some underlying mechanisms are exploited by TGEV to escape IFN-I-induced antiviral responses. Given that miR-30a-5p inhibited TGEV replication at the late stage of infection (Fig. 3H), we hypothesized that miR-30a-5p suppresses TGEV replication possibly by enhancing IFN-I antiviral signaling cascades rather than by promoting the production of IFN-I. To exclude the possibility that miR-30a-5p enhances the production of IFN-I, we initially analyzed IFN-β expression in ST cells following TGEV infection when overexpressing miR-30a-5p. Consistent with previous studies (8, 25), TGEV infection elicited a substantial amount of IFN-β production at 24 hpi (Fig. 4A). Overexpression of miR-30a-5p did not significantly increase IFN-β production, as measured by quantifying IFN-β protein levels following TGEV infection relative to those observed in the presence of NC mimics (Fig. 4A), indicating that miR-30a-5p does not modulate TGEV-induced IFN-I production.

The binding of IFN-I to the IFN-I receptor primarily activates the JAK-STAT1 signaling pathway and induces hundreds of ISGs to inhibit viral infection. To verify our hypothesis that miR-30a-5p hinders TGEV replication largely by enhancing IFN-I antiviral signaling cascades, we initially monitored the kinetic profiles of interferon-stimulated gene 15 (ISG15) expression post-TGEV infection (Fig. 4B). The expression of ISG15 peaked at 24 hpi and then significantly diminished, although IFN-β was continuously elevated during the period from 24 to 48 hpi (Fig. 4B) (P<0.05). Consistent with the kinetic profile of ISG15, STAT1 blotting results demonstrated that STAT1 phosphorylation peaked at 24 hpi and then decreased (Fig. 4C). These results suggest that IFN-I antiviral signaling cascades are undermined during the later stage of TGEV infection in contrast to IFN-β production. To directly verify if TGEV infection impairs IFN-I antiviral signaling at the later stage of infection, we stimulated TGEV-infected ST cells at 36 hpi by IFN-β for 30 min and evaluated STAT1 activation. Compared with uninfected control cells, TGEV-infected cells exhibited a significant reduction in IFN-β-elicited STAT1 phosphorylation (p-STAT1) (Fig. 4D), indicating that TGEV infection impairs IFN-I antiviral signaling at a later stage. Furthermore, the time frame of the observed decrease in ISG15 expression matched that of an observed significant reduction in miR-30a-5p abundance during TGEV infection (Fig. 4B), suggesting that miR-30a-5p may be relevant for the suppression of the IFN-I antiviral response at the later stage of infection. To validate the role of miR-30a-5p in modulating IFN-I antiviral signaling, we initially monitored the activity of the IFN-stimulated response element (ISRE) with a luciferase assay following IFN-β stimulation. Compared with NC mimics, MiR-30a-5p mimics significantly enhanced the activity of ISRE stimulated by IFN-β; in contrast, compared with the NC inhibitor, the miR-30a-5p inhibitor suppressed IFN-β-derived ISRE activity (Fig. 4E). We next measured the expression of antiviral ISG genes (ISG15, 2'-5'-oligoadenylate synthetase-like (OASL), and myxovirus resistance protein 1 (MxA)) in the presence of miR-30a-5p

overexpression during TGEV infection (Fig. 4F) or IFN-β stimulation (Fig. 4G). Compared the NC mimic control, MiR-30a-5p overexpression greatly upregulated the expression of antiviral ISG genes in response to either TGEV infection or IFN-β stimulation (Fig. 4F and 4G). Consistent with the results of ISRE luciferase and ISG expression assays, the overexpression of miR-30a-5p significantly promoted p-STAT1, whereas the inhibition of endogenous miR-30a-5p decreased p-STAT1 levels following IFN-β stimulation or TGEV infection (Fig. 4H). These data demonstrate that miR-30a-5p promotes IFN-I signaling. Overall, we conclude that miR-30a-5p reinforces IFN-I antiviral signaling rather than IFN-I production.

Consistent with previously published results (42, 43), we demonstrated that IFN-β substantially reduced TGEV infection and exhibited anti-TGEV activity *in vitro* (Fig. 4I). In agreement with the result that miR-30a-5p enhanced IFN-I antiviral signaling, compared with NC mimics, overexpression of miR-30a-5p reduced TGEV infection and increased the efficiency of IFN-β in inhibiting TGEV infection by more than 100-fold (Fig. 4I) (p<0.01). The inhibition of endogenous miR-30a-5p by the inhibitor significantly increased TGEV infection and decreased the efficiency of IFN-β in suppressing TGEV infection (Fig. 4I). Therefore, these data demonstrate that miR-30a-5p inhibits TGEV replication by enhancing IFN-I antiviral signaling rather than through the modulation of IFN-I production.

miR-30a-5p directly targets SOCS1 and SOCS3

To elucidate the underlying mechanisms by which miR-30a-5p enhances IFN-I signaling, we performed computational analysis by using a TargetScan prediction program to identify the potential target genes of miR-30a-5p. Since miR-30a-5p promoted IFN-I signaling rather than IFN-I induction (Fig. 4), we primarily focused on the target genes of miR-30a-5p that enhance IFN signaling pathways. Computational analysis showed that miR-30a-5p could potentially target SOCS1 and SOCS3 through a 3' UTR site that is conserved

in mammals (Fig. 5A). The SOCS family of proteins are endogenous potent negative regulators of JAK-STAT signaling transduction (19, 44). Next, we explored whether miR-30a-5p directly targets SOCS proteins and enhances IFN-I signaling by downregulating SOCS expression. To verify whether miR-30a-5p directly targets SOCS1 and SOCS3, we cloned the predicted target sites in the 3' UTRs of SOCS1 or SOCS3 into a firefly luciferase reporter vector. In addition, a mutant vector was constructed to eliminate possible recognition by replacing five seed nucleotides (UGUUUAC to UAGGGUC) as previously described (Fig. 5A) (30). Compared with the NC mimic treatment, overexpression of miR-30a-5p in ST cells decreased the activity of the luciferase reporter containing the SOCS1 or SOCS3 wild-type target sequence but not that of the luciferase reporter containing the mutant target site of SOCS1 or SOCS3. By contrast, compared with the NC inhibitor, the miR-30a-5p inhibitor increased the activity of the luciferase reporter containing the SOCS1 or SOCS3 wild-type target sequence but not that of the luciferase reporter containing the mutant target site (Fig. 5B). These results confirm that miR-30a-5p directly targets the 3' UTRs of SOCS1 and SOCS3. To further verify SOCS1 and SOCS3 as direct targets of miR-30a-5p, we examined the expression of SOCS1 and SOCS3 in ST cells transfected with miR-30a-5p mimics or inhibitor. As expected, miR-30a-5p mimics significantly decreased the transcript levels of SOCS1 and SOCS3 in ST cells (Fig. 5C). The diminished expression of SOCS1 and SOCS3 induced by miR-30a-5p overexpression was verified by Western blotting (Fig. 5D). Conversely, compared with the NC inhibitor, the miR-30a-5p inhibitor increased the expression of SOCS1 and SOCS3 in ST cells (Fig. 5C and 5D). The modulation of miR-30a-5p abundance exerted an effect on the expression of SOCS1 and SOCS3 in TGEV-infected ST cells similar to that in TGEV-uninfected ST cells (Fig. 5D). Altogether, these data demonstrate that miR-30a-5p downregulates the expression of SOCS1 and SOCS3 via directly targeting their 3' UTRs.

TGEV infection downregulated miR-30a-5p expression through activation of IRE1α (Fig. 1 and 2). Reasonably, TGEV infection would upregulate the expression of SOCS1 and SOCS3. As expected, TGEV infection substantially elevated the expression of SOCS1 and SOCS3 in ST cells at 24 hpi and exhibited a dose-dependent induction of MOIs (Fig. 5E). Furthermore, the increased expression of SOCS1 and SOCS3 began at 12 hpi and was substantially elevated at 24 hpi following TGEV infection as observed by measuring both mRNA and protein levels, which was inversely correlated with the kinetic expression profiles of miR-30a-5p (Fig. 5F and 5G). To further validate the role of miR-30a-5p in manipulating SOCS1 and SOCS3 expression via IRE1α, we assessed the expression of SOCS1 and SOCS3 after TGEV infection in the presence of 100 μM 4μ8c pretreatment. Compared with the DMSO-pretreated mock control, 4μ8c pretreatment strongly decreased the expression of SOCS1 and SOCS3 in ST cells following TGEV infection, and the reduction in SOCS1 and SOCS3 expression by 4μ8c was counteracted by the miR-30a-5p inhibitor (Fig. 5H), indicating that TGEV upregulates the expression of SOCS1 and SOCS3 through IRE1α-mediated modulation of miR-30a-5p expression. Consistent with the *in vitro* SOCS expression results, the expression of both SOCS1 (Fig. 5I) and SOCS3 (Fig. 5J) in TGEV-infected ileum was also upregulated more than 10-fold. Taken together, these data demonstrate that TGEV infection upregulates the expression of SOCS1 and SOCS3 via IRE1α-mediated modulation of miR-30a-5p abundance.

Increased expression of SOCS1 or SOCS3 disrupts the IFN-I antiviral response and facilitates TGEV replication

Because miR-30a-5p hindered TGEV infection (Fig. 3) and enhanced IFN-I signaling (Fig. 4), next, we explored whether TGEV-derived suppression of miR-30a-5p facilitates TGEV infection via modulation of IFN-I signaling by

SOCS1 and SOCS3. Initially, we analyzed the activation of the JAK-STAT1 pathway in response to IFN-I stimulation or TGEV infection of ST cells transfected with pCAGGS-SOCS1, pCAGGS-SOCS3, or empty vector. The expression of SOCS1 or SOCS3 was confirmed by an IFA (Fig. 6A). As expected, the overexpression of SOCS1 or SOCS3 reduced p-STAT1 and the ratio of p-STAT1 to total STAT1 in response to either IFN-β stimulation or TGEV infection (Fig. 6B), indicating that the elevated expression of SOCS1 or SOCS3 suppresses p-STAT1 induced by IFN-β or TGEV infection. Consistent with the disruption of IFN-I-activated JAK-STAT1 signaling by either SOCS1 or SOCS3, transient expression of SOCS1 substantially dampened the anti-TGEV activity of IFN-β and enhanced TGEV infection as indicated by quantification of TGEV genomes and viral titers, and the transient expression of SOCS3 also dampened the anti-TGEV activity of IFN-β but significantly elevated the levels of TGEV genomes only despite the trend of the increase in TGEV titers (Fig. 6C). Importantly, the overexpression of either SOCS1 or SOCS3 enhanced TGEV infection under physiological infection conditions in the absence of IFN-β stimulation (Fig. 6D). Overexpression of SOCS1 suppressed p-STAT1, IFN-β antiviral activity, and TGEV infection more efficiently than did overexpression of SOCS3 (Fig. 6B-6D). These results demonstrate that the increased expression of SOCS1 and SOCS3 hinders JAK-STAT1 signaling elicited by IFN-I or TGEV and enhances TGEV infection. To further verify the roles of SOCS1 and SOCS3 in IFN-I anti-TGEV activity, we silenced the expression of endogenous SOCS1 and SOCS3 using siRNAs. The knockdown efficiency of SOCS1 siRNAs and SOCS3 siRNAs was confirmed by Western blotting (Fig. 6E). By contrast, knockdown of endogenous SOCS1 or SOCS3 by specific siRNAs in ST cells substantially enhanced the anti-TGEV activity of IFN-β (Fig. 6F) and the expression of IFN-I-induced ISGs (Fig. 6G). Overall, the silencing of endogenous SOCS1 and SOCS3 expression greatly decreased TGEV titers by up to 10-fold (Fig. 6H) and enhanced the expression of IFN-induced ISGs (Fig. 6I) in

non-IFN-β-stimulated ST cells compared with those in siRNA NC control cells. In agreement with SOCS1 and SOCS3 overexpression results, the silencing of SOCS1 enhanced the IFN-β-mediated antiviral response and hindered TGEV infection more efficiently than did the knockdown of SOCS3 (Fig. 6E-6I), suggesting that SOCS1 has a more important role in IFN-I signaling than does SOCS3. Thus, these data collectively indicate that TGEV infection upregulates the expression of SOCS1 and SOCS3, which undermines JAK-STAT1 signaling and facilitates TGEV infection.

IRE1α facilitates TGEV infection by modulating the miR-30a-5p/SOCS axis

We demonstrated above that TGEV-activated IRE1α decreased the levels of miR-30a-5p (Fig. 2) and that miR-30a-5p directly targeted SOCS family proteins and downregulated their expression, which enhanced STAT1 activation and hindered TGEV infection (Figs. 3-6). To further verify whether TGEV escapes the IFN-I response by disrupting the JAK-STAT1 pathway through IRE1α-mediated modulation of the miR-30a-5p/SOCS axis, we initially monitored TGEV infection after inhibiting STAT1 with fludarabine (a STAT1-specific inhibitor) in ST cells treated with 4μ8c. The suppression of IRE1α by 4μ8c promoted STAT1 activation, resulting in TGEV inhibition (Fig. 7A). The disruption of 4μ8c-mediated enhancement of STAT1 signaling by fludarabine significantly rescued TGEV suppression by 4μ8c (Fig. 7A). In agreement with these results, the overexpression of miR-30a-5p enhanced STAT1 activation and hindered TGEV infection, and fludarabine-mediated blockade of STAT1 activation significantly rescued the suppression of TGEV infection by miR-30a-5p (Fig. 7C). The inhibition of STAT1 activation by fludarabine was confirmed by Western blotting for p-STAT1 and total STAT1 (Fig. 7B and 7D). The rescue of 4μ8c or miR-30a-5p-mediated TGEV inhibition by fludarabine treatment was not due to cellular cytotoxicity since treatment with 4μ8c, fludarabine, or 4μ8c plus fludarabine did not cause

cellular cytotoxicity at the concentrations used in the study (Fig. 7E). These results suggest that IRE1α enhances TGEV infection through miR-30a-5p/SOCS-mediated disruption of the JAK-STAT1 pathway. Finally, to directly verify whether the effect of the miR-30a-5p/SOCS axis on TGEV infection is dependent on the IFN-I antiviral response, we assessed the suppression of miR-30a-5p on TGEV in ST cells after knockdown of the IFN-I receptor IFNAR1. The silencing efficacy of IFNAR1 siRNAs was verified by measuring transcripts of IFNAR (Fig. 7F). Among the 3 tested siRNAs, only siIFNAR1#1 substantially silenced IFNAR1 expression (Fig. 7F). The silencing of IFNAR1 by siIFNAR1#1 compared with that by the siRNA control was further confirmed by the reduction in ISG expression (ISG15, OASL, and MxA) after IFN-β stimulation (Fig. 7G). Unlike the siRNA control, no inhibitory effect of miR-30a-5p on TGEV was observed in IFNAR1-slienced cells (Fig. 7H). In line with TGEV titer results, miR-30a-5p overexpression did not result in the enhancement of STAT1 activation in IFNAR1-silenced ST cells, whereas miR-30a-5p overexpression did in wild-type ST cells (Fig. 7I). These data demonstrate that miR-30a-5p overexpression suppresses TGEV infection via the IFN-I response. In summary, IRE1α enhances TGEV infection through miR-30a-5p/SOCS axis-mediated escape from the IFN-I response.

Discussion

IFN-I has vital roles in the host innate immunity response against viral infections, and in turn, coronaviruses have evolved diverse strategies to counter the antiviral activity of IFN-I during infection (20, 23, 45). Coronavirus replication is structurally and functionally related to the ER, and ER stress is a common outcome in coronavirus-infected cells (1, 2, 45). The UPR constitutes a vital aspect of the virus-host interaction and modulates both viral replication and pathogenesis. However, an understanding of how UPR induction by coronaviruses actively participates in viral replication and manipulates the host

innate immune responses has remained elusive. In the present study, we observed that TGEV infection induced the activation of IRE1α, which facilitated TGEV infection by modulating the miR-30a-5p/SOCS axis. Our results revealed an unappreciated mechanism employed by TGEV to escape the IFN-I antiviral response via IRE1α-mediated modulation of the miR-30a-5p/SOCS axis (Fig. 8).

IRE1α, the most conserved UPR transducer with both kinase and RNase activities, plays a critical role in restoring ER homeostasis (38, 46). Many viruses have evolved strategies to employ IRE1α signaling to facilitate their infection. The inhibition of IRE1α signaling by specific IRE1α inhibitors or siRNA decreases the replication of IAV, JEV and hepatitis C virus (HCV) (17, 18, 47). We also showed that the inhibition of IRE1α by the specific inhibitor 4μ8c or by siRNA knockdown diminished TGEV replication *in vitro,* similar to that observed for IAV, JEV and HCV. However, the mechanisms by which IRE1α facilitates viral infection remain elusive. IRE1α may facilitate viral infection by conferring resistance of the infected cells to apoptosis, as observed for HCV (47). IRE1α facilitates JEV replication by modulating viral RNA translation through the RIDD pathway (18). In this study, we identified another mechanism of IRE1α enhancement of viral infection, which occurs through reducing the endogenous abundance of miR-30a-5p. We also observed that IRE1α mediated the decrease in miR-125b and miR-125a levels in Tg-treated ST cells (data not shown). Thus, the downregulation of miR-30a-5p by IRE1α is not specific to miR-30a-5p. This finding is consistent with previous studies reporting that IRE1α manipulates the expression of subsets of cellular miRNAs, including miR-125b, miR-150, and miR-17, in response to ER stress (15, 38, 47). However, the specificity and mechanisms of IRE1α-mediated manipulation of cellular miRNAs remain elusive and merit further study.

In contrast to PEDV, which inhibits dsRNA-mediated IFN-I production (20), TGEV infection results in very rapid and massive production of IFN-I *in vitro*

and *in vivo* (26, 27, 43, 48). Because TGEV infection is sensitive to IFN-I activity, TGEV has several strategies to antagonize IFN-I signaling rather than IFN-I production. This information is consistent with our finding that TGEV impairs IFN-I JAK-STAT1 signaling at the late stage of infection (Fig. 4B-4D). SOCS family proteins are negative regulators of cytokine-mediated JAK-STAT signaling. Here, TGEV infection inhibited miR-30a-5p expression, resulting in strongly upregulated expression of SOCS1 and SOCS3 (Figs. 1 and 5), allowing efficient TGEV replication despite high IFN-I levels (Fig. 6). Knockdown of the physiological expression levels of SOCS1 or SOCS3 resulted in up to a 4-fold increase in the expression of ISG15 and a more than a 200-fold increase in the anti-TGEV activity of IFN-I (Fig. 6), indicating that SOCS3 and especially SOCS1, which suppresses IFN-I signaling more strongly than SOCS3, play vital roles in negatively regulating the IFN-I response during TGEV infection. Our data indicate that TGEV largely evades IFN-I innate immunity largely by modulating IFN-I antiviral signaling rather than by manipulating the production of IFN-I. The induction of SOCS1 and SOCS3 to counteract IFN antiviral responses is also observed in other viruses, such as SARS-CoV (49), herpes simplex virus type 1 (50), respiratory syncytial virus (51), HCV (31), and IAV (52). SOCS1 and SOCS3 act on multiple STAT family members and potently suppress both JAK-STAT1 and JAK-STAT3 signaling pathways (53, 54). Thus, our study provides more evidence that SOCS family proteins play a crucial role in antagonizing IFN-I responses during viral infections.

Our results showed that miR-30a-5p enhanced IFN-I signaling and significantly suppressed viral infection by directly targeting SOCS1 and SOCS3 whose 3' UTRs are conserved in mammals (Fig. 5). Consistent with our data, during the preparation of this article, a paper published by Zhen Xu and colleagues revealed that miR-30a-5p directly targets the 3' UTR of SOCS3 and manipulates the expression of SOCS3 in mouse B cell lymphoma (55). Importantly, we detected decreased expression of miR-30a-5p and increased

expression of SOCS1 and SOCS3 in TGEV-infected ileum. Reasonably, we speculate that the miR-30a-5p/SOCS axis may have a role in TGEV pathogenesis *in vivo*. Given that activation of IRE1α and upregulation of SOCS family proteins by many other viruses are not uncommon, the IRE1α-miR-30a-5p-SOCS axis may also be involved in the pathogenesis of other viral infections, and further study is worthwhile.

In conclusion, here, we report a previously unappreciated mechanism by which TGEV escapes IFN antiviral activity via IRE1α-mediated miR-30a-5p suppression and subsequent SOCS1 and SOCS3 upregulation (Fig. 8). Our findings underscore the importance of IRE1α in the regulation of IFN-I signaling and TGEV infection, as well as broaden our knowledge regarding the role of the UPR in aberrant host miRNA expression following viral infection. In addition, our study sheds light on the crucial roles of SOCS1 and SOCS3 in coronavirus infection.

Materials and Methods

Cell culture, viruses and virus infection. ST cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in an incubator with 5% CO_2 at 37°C. TGEV strain H87, derived from the virulent strain H16 (GenBank: FJ755618), was propagated in ST cells. A TGEV stock was prepared and titrated as previously described (56). For TGEV infection, ST cells were infected with TGEV H87 at a desired MOI or mock infected with DMEM. After a 2-h incubation at 37°C, cells were washed and cultured in DMEM supplemented with 1% DMSO and 0.3% trypsin (0.25%, Gibco, USA) until harvested.

Synthetic miRNAs, siRNAs, and transfection. All miRNA mimics, miRNA inhibitors, and siRNAs listed in Table 1 were designed and synthesized by GenePharma (China). Cells were seeded for 16 h before transfection with plasmid DNA or synthetic oligonucleotides using Lipofectamine 2000

(Invitrogen, USA) or Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. The cells were infected with TGEV or stimulated by IFN-β at 24 h after transfection. The cells were harvested for quantitative real-time reverse transcription PCR (RT-qPCR) or Western blotting after a 24-h incubation.

Chemical treatments. Thapsigargin (Tg, a potent inducer of ER stress) and 4μ8c (a specific inhibitor of IRE1α) (14) were purchased from Sigma-Aldrich, and fludarabine (a STAT1 specific inhibitor) was purchased from Selleck. ST cells were pretreated with different concentrations of chemicals or DMSO for 2 or 24 h, followed by inoculation with TGEV. After a 2-h incubation, the supernatant was removed and replaced with culture media containing different doses of chemicals. The samples were harvested for RT-qPCR and viral titration at 24 hpi.

RNA extraction, reverse transcription, and qPCR. Total cellular RNA was extracted using TRIzol (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. cDNA was prepared with a PrimeScript™ II 1st strand cDNA synthesis kit (Takara, Japan). The expression pattern of each gene was analyzed by RT-qPCR using a LightCycler480 II system (Roche, Swiss) as previously described (56). For miRNA analyses, RNA was reverse transcribed by using a miRNA First Strand cDNA Synthesis kit (Sangon Biotech, China), and miRNA expression was assessed by RT-qPCR. PCR amplification was performed in triplicate using the following conditions: 95°C for 10 min, followed by 40 cycles of two steps (95°C for 15 s and 60°C for 40 s). The relative expression levels of miRNAs were normalized to those of U6. The RNA expression results are presented as the means±standard error of the mean (SEM) from 3 independent experiments. The sequences of RT-qPCR primers are listed in Table 2.

microRNA target prediction and plasmid construction. Target Scan 7.1 (http://www. targetscan.org) was used to predict SOCS1 and SOCS3 3' UTRs as potential targets of miR-30a-5p. The 3' UTR of porcine SOCS1 (**GenBank:**

GQ421919.1) or SOCS3 (**GenBank: AY785557.1)** was amplified and inserted into the pmirGLO luciferase reporter vector (Promega, USA) using Nhe I and Xba I restriction sites. The mutant types of SOCS1 or SOCS3 3' UTR vectors were constructed by mutating five seed nucleotides using a site-directed mutagenesis kit (Stratagene, USA) according to the manufacturer's instructions. To construct the SOCS1 and SOCS3 expression vectors, we amplified the full-length CDS regions by specific primers, and the amplicons were cloned into the vector pCAGGS-HA (Addgene) using EcoR I and Kpn I restriction sites. The porcine ISRE reporter plasmid was kindly provided by Prof. Wenhai Feng from China Agricultural University and was previously described (30). All primers are listed in Table 2.

Dual-luciferase reporter assays. For miRNA target verification, wild-type or mutant SOCS1 and SOCS3 3' UTR luciferase reporter vectors were cotransfected with miR-30a-5p mimics, mimic NC, miR-30a-5p inhibitor, or inhibitor NC (NC-i) into ST cells for 36 h. Furthermore, the ISRE reporter plasmid and pRL-TK were cotransfected with either miR-30a-5p mimics, mimic NC, inhibitor, or inhibitor NC for 24 h, followed by incubation with porcine IFN-β (100 ng/mL) for 12 h. Next, the cells were collected for reporter activity testing with a Dual-Luciferase Reporter Assay System (Promega, USA), according to the manufacturer's protocol. All reporter assays were independently repeated at least three times.

Immunofluorescence assay (IFA). IFAs were performed as previously described (57). Briefly, ST cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 and then blocked with blocking buffer for 2 h at 37°C. Cells were incubated with an anti-TGEV nucleocapsid monoclonal antibody (1:1000) stocked in our laboratory or an anti-HA monoclonal antibody (Sigma-Aldrich, 1:5000) at 37°C for 2 h. The cells were then labeled with an Alexa Fluor 546 goat anti-mouse immunoglobulin G (IgG) antibody (1:500) (Thermo Fisher Scientific, USA) for 1 h at 37°C. Cellular nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:100). Stained cells were

visualized using an AMG EVOS F1 florescence microscope.

ELISA. Culture supernatants were collected from treated cells at the indicated times and stored at -80°C until testing. IFN-β protein levels in culture supernatants were determined by porcine IFN-β enzyme-linked immunosorbent assay (ELISA) kits (Bio-Swamp, China) according to the manufacturer's instructions.

Protein extraction and Western blotting. To analyze the levels of host IRE1α, phospho-STAT1, STAT1, SOCS1, SOCS3 proteins, we analyzed the cellular lysate by Western blotting as previously described (56). Briefly, ST cells were lysed with Nonidet P-40 (NP-40) lysis buffer (Beyotime, China) supplemented with cocktail protease inhibitor (Roche). Protein concentrations of the supernatant fraction were measured with a bicinchoninic acid (BCA) assay kit (Beyotime, China) and were equalized with extraction reagent. Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto a nitrocellulose membrane (GE Healthcare, USA). Membranes were blocked and incubated with the corresponding primary antibody at 4°C overnight. The primary antibodies used are as follows: β-actin (Sigma-Aldrich, 1:5000), SOCS1 (Sigma-Aldrich, 1:500), SOCS3 (CST, 1:1000), phospho-STAT1 (Abcam, 1:1000), STAT1 (Abcam, 1:1000) and IRE1α (Santa Cruz Biotechnology, 1:500). After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG for 1 h at room temperature. Then, immunolabeled proteins were visualized using electrochemiluminescence (ECL) reagent (Thermo Fisher Scientific). The intensity of each band was analyzed using ImageJ.

TGEV infection of piglets. Twelve two-day-old specific pathogen free (SPF) piglets were randomly divided into two groups. Piglets in group one were orally inoculated with 5 mL 1×10^5 $TCID_{50}$ TGEV H87 strain. After viral infection, clinical signs were recorded on a daily basis. All piglets were euthanized at 48 hpi. Piglet small intestine samples were collected for RT-qPCR analyses. The

TGEV infection experiment was approved by the Animal Care and Ethics Committee of Harbin veterinary research institute.

Cellular cytotoxicity assay. ST cells were cultured in 96-well plates for 24 h, followed by incubation with Tg, 4μ8c, fludarabine, 4μ8c plus fludarabine, or the same volume of DMSO for 24 h or 48 h. Next, cellular cytotoxicity assays were performed using a Cell Counting Kit-8 (CCK-8) assay as previously described (56).

Statistical analysis. All results in figures are presented, where appropriate, as the means±SEM from three independent experiments and were analyzed in GraphPad Prism (GraphPad Software, Inc). Differences were considered significant if the *p* value was <0.05. The *p* values are indicated as follows: **p*<0.05, ***p*<0.01, ****p*<0.001.

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852 **Table 1 Sequences of miRNA mimics, inhibitors, and siRNAs**

Small RNA	Sequence (5'-3')
miR-30a-5p	UGUAAACAUCCUCGACUGGAAG
miR-30a-5p inhibitor	CUUCCAGUCGAGGAUGUUUACA
siIRE1α#1	GCACAGACCUGAAGUUCAATT
siIRE1α#2	GGAGGUUAUCGACCUGGUUTT
siIRE1α#3	CCAUCAUCCUGAGCACCUUTT
siSOCS1#1	CCUGCACGGAGCAUUAACUTT
siSOCS1#2	UCUUCGCCCUCAGUGUGAATT
siSOCS1#3	GCCGACAAUGCAAUCUCCATT
siSOCS3#1	UCAAGCUGGUGCGUCACUATT
siSOCS3#2	CCUGGACUCCUAUGAGAAATT
siSOCS3#3	UCUUCACGCUCAGCGUCAATT
siIFNAR1#1	CCAGCUUUACCCACUAAUUTT
siIFNAR1#2	CCGGGUCUAUGUUCUUAAATT
siIFNAR1#3	GCCUGGAUGUCAAUAUGUUTT

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866 **Table 2 Sequences of primers used in the present study**

Primer	Sequences (5'-3')
miR-30a-5p-qPCR-F	TGTAAACATCCTCGACTGGAAG
Uni-miR-qPCR-R	GCGAGCACAGAATTAATACGACTCAC
ISG15-qPCR-F	AGC ATG GTC CTG TTG ATG GTG
ISG15-qPCR-R	CAG AAA TGG TCA GCT TGC ACG
MxA-qPCR-F	CACTGCTTTGATACAAGGAGAGG
MxA-qPCR-R	GCACTCCATCTGCAGAACTCAT
OASL-qPCR-F	TCCCTGGGAAGAATGTGCAG
OASL-qPCR-R	CCCTGGCAAGAGCATAGTGT
SOCS1-qPCR-F	CGCCCTCAGTGTGAAGATGG
SOCS1-qPCR-R	GCTCGAAGAGGCAGTCGAAG
SOCS3-qPCR-F	CACTCTCCAGCATCTCTGTC
SOCS3-qPCR-R	TCGTACTGGTCCAGGAACTC
TGEV-qPCR-F	GCTTGATGAATTGAGTGCTGATG
TGEV-qPCR-R	CCTAACCTCGGCTTGTCTGG
GAPDH-qPCR-F	CCTTCCGTGTCCCTACTGCCAAC
GAPDH-qPCR-R	GACGCCTGCTTCACCACCTTCT
IFN-β-qPCR-F	AGCACTGGCTGGAATGAAAC
IFN-β-qPCR-R	TCCAGGATTGTCTCCAGGTC
ERdj4-qPCR-F	CAGAGAGATTGCAGAAGCATATGA
ERdj4-qPCR-R	GCTTCTTGGATCGAGTGTTTTG
IFNAR1-qPCR-F	ACATCACCTGCCTTCACCAG
IFNAR1-qPCR-R	CATGGAGCCACTGAGCTTGA
XBP1-F	AAACAGAGTAGCAGCTCAGACTGC
XBP1-R	GAATCTCTAAGACTAGGGGCTTTGTA
SOCS1-EcoR I–F	CGGAATTCATGGTAGCACACAACCAGGTG
SOCS1-Kpn I–R	GGGGTACCTCATATCTGGAAGGGGAAGGAG
SOCS3-EcoR I–F	CGGAATTCATGGTCACCCACAGCAAGTTC
SOCS3-Kpn I–R	GGGGTACCTTAAAGTGGGGCATCGTACTGC
SOCS1-3' UTR-Nhe I-F	CTAGCTAGCATTATTTCCTTGGAACCATGTG
SOCS1-3' UTR-Xba I-R	GCTCTAGACACAGCAGAAAAATAAAGCCAG
SOCS3-3' UTR-Nhe I-F	CTAGCTAGCTTCTATTTTGTGCCTCCTGAC
SOCS3-3' UTR-Xba I-R	GCTCTAGAGTTTGACTTGGATTGGTATTTC
SOCS1-3' UTR-MT–F	CTTCATAGGGTCATATACCCAGTATCTTTGCACAAAC
SOCS1-3' UTR-MT–R	TATATGACCCTATGAAGAGGTAGGAGGTACTGAGTTC
SOCS3-3' UTR-MT–F	ATAATAGGGTCAATCTGCCTCAATCACTCTGTCTTTTA
SOCS3-3' UTR-MT–R	TTGACCCTATTATTAAAAAACACAAACAAAACCCAAAC

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869 **Figure legends**

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871 **Fig. 1.TGEV infection suppresses miR-30a-5p expression *in vitro* and *in**

872 ***vivo*.** (A). The ER stress inducer Tg decreased miR-30a-5p expression in ST

873 cells. The miR-30a-5p levels in ST cells were measured by RT-qPCR after Tg

874 treatment for 24 h. (B-E). TGEV infection downregulated miR-30a-5p

875 expression *in vitro*. The miR-30a-5p levels (B) and TGEV infection (C) in ST

876 cells were measured by RT-qPCR at 24 hpi with different MOIs. For time

877 kinetics, the levels of miR-30a-5p (D) and TGEV genomes (E) in ST cells were

878 quantified at the indicated time points after infection with TGEV at a MOI of 1.

879 The results from three independent experiments are shown. (F, G). TGEV

880 infection suppressed miR-30a-5p expression *in vivo*. Piglets were orally

881 inoculated with 5 mL 1×10^5 $TCID_{50}$ TGEV or DMEM. Total cellular RNA from

882 each ileum was collected at 48 hpi, and the levels of miR-30a-5p (F) and

883 TGEV viral RNA in ileum (G) were measured by RT-qPCR. *, $p<0.05$; **,

884 $p<0.01$; ***, $p<0.001$ vs. mock-infected control.

885 **Fig. 2. Activated IRE1α reduces miR-30a-5p abundance.** (A). Analysis of

886 IRE1α activation by XBP1 mRNA splicing. IRE1α activation by TGEV infection

887 was analyzed by PCR amplification of total XBP1 cDNA and further digestion

888 with Pst1 as previously described (40). The sizes of the PCR-amplified

889 fragments from spliced and unspliced XBP1 DNA with or without PstI cleavage

890 are also listed. The PCR fragments of total XBP1, spliced and unspliced XBP1

891 DNA in ST cells that were infected with TGEV at a MOI of 1 for various time

892 points or treated with Tg (1 μM) for 24 h are shown. The PCR products of the

893 housekeeping gene GAPDH are shown in the top panel as an internal control.

894 The ratio of the band intensities for spliced and total XBP1 DNA in the infected

895 cells was normalized to that of the mock-infected cells. (B). TGEV infection

896 upregulated ERdj4 expression. The relative expression of ERdj4 to GAPDH

897 following TGEV infection was measured and presented. (C). Analysis of IRE1α

898 activation in partial samples from A, D, or E. (D-E). The inhibition of IRE1α by

4μ8c rescued the suppression of miR-30a-5p by TGEV (D) or Tg (E). ST cells were pretreated with 50 or 100 μM 4μ8c for 2 h followed by TGEV infection (MOI=1) (D) or Tg (1 μM) (E). The relative expression of miR-30a-5p to internal U6 siRNA was measured by RT-qPCR after 24 h. The results are presented as the relative expression of miR-30a-5p in ST cells normalized to that of miR-30a-5p in the mock control cells. (F-H). Knockdown of IRE1α rescued miR-30a-5p suppression following TGEV infection or Tg treatment. ST cells were transfected with siIRE1α#1, siIRE1α#2, siIRE1α#3, or siRNA scrambled control at 100 nM for 24 h, followed by infection with TGEV for 24 h at a MOI of 0.01 (F and G) or treatment with Tg (1 μM) for 24 h (H). Next, the cells were harvested to determine the efficiency of IRE1α knockdown (F) or miR-30a-5p expression (G, H). The results represent three independent experiments.

Fig. 3. miR-30a-5p inhibits the replication of TGEV. (A-B). The inhibition of IRE1α by 4μ8c suppressed TGEV infection (MOI=1) in ST cells. ST cells were pretreated with 50 or 100 μM 4μ8c for 2 h and then infected with TGEV (MOI=1); TGEV viral RNA (A) and titers (B) were measured at 24 hpi. (C-D). IRE1α knockdown by siRNAs decreased TGEV replication. ST cells were transfected with IRE1α siRNA or siRNA scrambled control at 100 nM, followed by infection with TGEV (MOI=0.01); TGEV viral RNA (C) and titers (D) were quantified at 24 hpi. *, $p<0.05$, **, $p<0.01$ vs. siRNA NC. (E-G). miR-30a-5p overexpression inhibited TGEV infection. ST cells were transfected with miR-30a-5p mimics at the indicated doses (20, 80 and 160 nM) for 24 h, followed by infection with TGEV for 24 h at a MOI of 0.01. TGEV infection was determined at 24 hpi by RT-qPCR (E) or titration (F). *, $p<0.05$, ***, $p<0.001$ vs. mimic NC. (G). The suppression of TGEV infection by miR-30a-5p was confirmed by IFA. (H). miR-30a-5p suppressed TGEV replication at the late stages of infection. ST cells were transfected with 160 nM miR-30a-5p mimics for 24 h and then infected with TGEV at a MOI of 0.01. TGEV infection was analyzed at 2, 6, 12, 24, or 36 hpi. (I). The miR-30a-5p inhibitor rescued TGEV suppression by 4μ8c. ST cells were transfected with 160 nM miR-30a-5p

929 inhibitor or NC inhibitor. After 24 h of transfection, ST cells were treated with
930 100 μM 4μ8c for 2 h and then infected with TGEV (MOI=1). TGEV infection
931 was measured at 24 hpi.

932 **Fig. 4. miR-30a-5p enhances IFN-I antiviral signaling rather than IFN-I**
933 **production.** (A). miR-30a-5p did not manipulate IFN-β production. ST cells
934 were transfected with 160 nM miR-30a-5p mimics or NC mimics for 24 h,
935 followed by infection with TGEV (MOI of 0.01) for 24 h. The IFN-β levels in
936 supernatant were measured by ELISA. (B-C). TGEV infection antagonized
937 interferon signaling at the late stages of infection. ST cells were infected with
938 TGEV at a MOI of 1, and then the samples were collected at different times for
939 the quantification of ISG15, IFN-β and miR-30a-5p expression (B) or the
940 Western blotting analysis of pSTAT1, STAT1 or β-actin (C). (D). TGEV infection
941 impaired IFN-I elicited STAT1 signaling. ST cells were infected with TGEV at a
942 MOI of 1 for 36 h, followed by stimulation with IFN-β for 30 min. Then, cells
943 were lysed and collected for Western blotting analysis of pSTAT1, STAT1 or
944 β-actin. (E). miR-30a-5p modulated the activity of the ISRE reporter vector
945 following IFN-β stimulation. The ISRE reporter vector and pRL-TK were
946 cotransfected with the indicated miR-30a-5p mimics, NC mimics, miR-30a-5p
947 inhibitor, or NC inhibitor into ST cells for 24 h, followed by stimulation with
948 IFN-β (100 ng/mL). Cells were harvested for luciferase assay at 12 h after
949 IFN-β stimulation. (F-H). miR-30a-5p promoted IFN-β signaling. The cells were
950 transfected with 160 nM miR-30a-5p mimics, NC mimics, miR-30a-5p inhibitor,
951 or NC inhibitor for 24 h, followed by IFN-β treatment or TGEV infection
952 (MOI=1). The samples were collected at 24 h after TGEV infection or IFN-β
953 stimulation for the quantification of ISG expression (F, G) and for Western
954 blotting analysis of pSTAT1, STAT1 or β-actin (H). Quantifications were
955 normalized to the mock-treated and uninfected NC control. (I). miR-30a-5p
956 enhanced the anti-TGEV activity of IFN-β. After transfection with miR-30a-5p
957 mimics or inhibitor for 24 h, cells were pretreated with IFN-β or DMEM for 24 h
958 and then infected with TGEV (MOI=0.01) and harvested at 24 hpi for viral RNA

959 quantification.

960 **Fig. 5. miR-30a-5p targets the 3' UTRs of SOCS1 and SOCS3.** (A).
961 Schematic diagram of the predicted target sites of miR-30a-5p in SOCS1 and
962 SOCS3 3' UTRs of six representative mammals. The predicted target sites and
963 mutated target sites of miR-30a-5p are underlined and were mutated as
964 indicated. (B). The results of the luciferase assay. ST cells were cotransfected
965 with SOCS1 or SOCS3 wild-type or mutant luciferase vectors (500 ng), and
966 160 nM miR-30a-5p mimics or NC mimics, miR-30a-5p inhibitor or NC inhibitor,
967 and the luciferase activity was analyzed at 30 h after transfection. (C). The
968 suppression of SOCS1 and SOCS3 mRNA levels by miR-30a-5p under
969 TGEV-uninfected conditions. ST cells were transfected with 160 nM NC
970 mimics, miR-30a-5p mimics, NC inhibitor, or miR-30a-5p inhibitor. The
971 expression levels of SOCS1 and SOCS3 were analyzed by RT-qPCR at 48 h
972 after transfection. The relative expression of SOCS1 and SOCS3 was
973 normalized to that of the NC control. Bars represent the means±SEM (n=3).
974 (D). The suppression of SOCS1 and SOCS3 protein levels by miR-30a-5p
975 under TGEV-uninfected and infected conditions. ST cells were transfected as
976 described in panel C for 24 h, followed by infection with TGEV (MOI=1) or
977 mock DMEM, and the samples were collected at 24 h for Western blotting
978 analysis of SOCS1, SOCS3 or β-actin. Quantifications were normalized to the
979 uninfected NC control. (E-G) TGEV infection upregulated SOCS1 and SOCS3
980 expression. The SOCS1 and SOCS3 expression levels (E) in ST cells were
981 measured by RT-qPCR at 24 hpi with different MOIs. *p* represents the
982 difference vs. the mock-infected control. For time kinetics, the SOCS1 and
983 SOCS3 mRNA levels (F) or SOCS1 and SOCS3 protein levels (G) in ST cells
984 were measured at indicated time points after infection with TGEV at a MOI of 1.
985 (H). Treatment with 4μ8c abolished the upregulation of SOCS1 and SOCS3 by
986 TGEV infection via modulating miR-30a-5p. ST cells were transfected with 160
987 nM miR-30a-5p inhibitor for 24 h. Next, ST cells were pretreated with 100 μM
988 4μ8c or DMSO for 2 h followed by infection with TGEV (MOI=1). Cells were

collected for RT-qPCR analysis for SOCS1 and SOCS3 expression at 24 hpi. (I-J) Elevated expression of SOCS1 and SOCS3 in the ilea after TGEV infection. The expression of SOCS1 (I) and SOCS3 (J) in the ilea at 48 hpi was quantified by RT-qPCR. Bars represent the means±SEM.

Fig. 6. Increased expression of SOCS1 or SOCS3 dampens the IFN-I antiviral response and promotes TGEV replication. (A). Overexpression of SOCS1 and SOCS3 in ST cells. ST cells were transfected as indicated with pCAGGS-HA, pCAGGS-SOCS1 or pCAGGS-SOCS3 for 48 h, and the transient expression of SOCS1 and SOCS3 was confirmed by IFA with anti-HA staining. (B). SOCS1 or SOCS3 overexpression suppressed the activation of STAT1 by IFN-β and TGEV infection. ST cells were transfected as indicated with pCAGGS-HA, pCAGGS-SOCS1, or pCAGGS-SOCS3 for 24 h, followed by incubation with porcine IFN-β (100 ng/mL) or infection with TGEV (MOI=1). Cells were collected for Western blotting analysis of pSTAT1, STAT1 or β-actin after 24 h. *p* represents the difference vs. the vector control. (C-D). SOCS1 or SOCS3 overexpression enhanced TGEV infection and undermined the anti-TGEV activity of IFN-β. ST cells were transfected as described in panel B, followed by incubation with porcine IFN-β (C) or DMEM (D) for 24 h. Then, cells were infected with TGEV at a MOI of 0.01; TGEV infection was determined at 24 hpi. (E). Knockdown of SOCS1 and SOCS3 by siRNAs in ST cells. ST cells were harvested for Western blotting analysis of SOCS1 and SOCS3 expression at 48 h after transfection with 100 nM siSOCS1s, siSOCS3s, or scrambled control siRNA. (F). Enhancement of anti-TGEV activity of IFN-β by knockdown of SOCS1 or SOCS3 in ST. ST cells were transfected with siSOCS1s, siSOCS3s, or the scrambled control for 24 h, followed by incubation with IFN-β for 24 h. Then, cells were infected with TGEV (MOI=0.01) and harvested for quantification of TGEV infection at 24 hpi. (G) Silencing of SOCS1 or SOCS3 boosted IFN-β signaling under IFN-β-stimulated conditions. ST cells were stimulated with IFN-β at 24 h after transfection with siSOCS1#1, siSOCS3#3, or the scrambled control, and cells

were collected for RT-qPCR analysis of ISG15, OASL, or MxA expression relative to that of GAPDH after 24 h stimulation. (H) Silencing of SOCS1 or SOCS3 decreased TGEV infection under IFN-β unstimulated conditions. ST cells were transfected with siSOCS1#1, siSOCS3#3, or the scrambled control for 24 h. Then, cells were infected TGEV (MOI=0.01) and harvested for quantification of TGEV infection at 24 hpi. (I) Silencing of SOCS1 or SOCS3 boosted IFN-β signaling under TGEV-infected conditions. ST cells were treated as described in Panel H, and cells were collected for RT-qPCR analysis of ISG15, OASL, or MxA expression relative to that of GAPDH.

Fig. 7. IRE1α facilitates TGEV infection by modulating the miR-30a-5p/SOCS axis

(A-B) The blockage of STAT1 activation rescued the viral suppression of 4μ8c. ST cells were pretreated with 100 μM 4μ8c for 2 h or 100 μM 4μ8c for 2 h plus 10 μM fludarabine for 24 h, followed by infection with TGEV (MOI=1). Then, the TGEV titer (A) was measured at 24 hpi, and STAT1 and p-STAT1 were analyzed by Western blotting (B). (C-D). The blockage of STAT1 activation rescued the viral suppression of miR-30a-5p. ST cells were transfected with 160 nM NC mimics, miR-30a-5p mimics or miR-30a-5p mimics plus 10 μM fludarabine stimulation for 24 h, followed by infection with TGEV (MOI=1). Next, the TGEV titer (C) was measured at 24 hpi, and STAT1 and p-STAT1 were analyzed by Western blotting (D). (E) Effect of 4μ8c and fludarabine on cell viability. ST cells were treated with 4μ8c, 4μ8c plus fludarabine, fludarabine only, or the carrier control DMSO as described above. Cell cytotoxicity was analyzed with a CCK-8 system as described in the Materials and Methods. (F) Knockdown of IFNAR1 by siRNAs in ST cells. ST cells were harvested for RT-qPCR analysis of IFNAR1 expression at 48 h after transfection with 100 nM siRNAs or scrambled control siRNA. (G) Silencing of IFNAR1 dampened IFN-β signaling under IFN-β-stimulated conditions. ST cells were transfected with 100 nM siIFNAR1#1 or scrambled control siRNA for 24 h, followed by incubation with IFN-β for 24 h. Then, cells were collected for RT-qPCR

analysis of ISG15, OASL, or MxA expression relative to that of GAPDH. (H and I). Silencing of IFNAR1 abolished the viral suppression (H) and enhancement of p-STAT1 (I) of miR-30a-5p. NC mimics or miR-30a-5p mimics (160 nM) were cotransfected with 100 nM siIFNAR1#1 or the scrambled control in ST cells for 24 h, followed by infection with TGEV (MOI=1). The TGEV titer (H), STAT1 and p-STAT1 (I) were measured at 24 hpi.

Fig. 8. TGEV antagonizes IFN-I-related innate immunity via IRE1α-mediated manipulation of the miR-30a-5p-SOCS axis. During TGEV infection, TGEV activates IRE1α, which reduces miR-30a-5p abundance. The decreased level of miR-30a-5p dampens IFN-I antiviral signaling by increasing the expression of SOCS1 and SOCS3, leading to viral escape from the IFN-I response.